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Application of statistical design in medium optimization for α - amylase production by *Bacillus subtilis* HB04

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ABSTRACT:
To optimize the medium conditions for maximum production of α - amylase by *Bacillus subtilis* HB04 in submerged fermentation, statistical design: Plackett - Burman Design and Central Composite Design were employed. The results of PBD statistical factorial design showed that in the culture medium, inoculum concentration, incubation time and yeast extract were the key factors affecting α - amylase production. The optimum cultural conditions derived from CCD for α - amylase production were, inoculum concentration 3%, incubation time 33h and yeast extract 3 g/L. The maximum production of 292.34 U/mL α - amylase was achieved under the optimum conditions. The results are encouraging for α - amylase production under CCD optimization for further pilot scale or industrial scale study.

Key words: Amylase; Optimization; Central Composite Design; *Bacillus subtilis* HB04

INTRODUCTION

Amylases are enzymes which hydrolyse starch molecules to give diverse products including dextrins and progressively smaller polymers composed of glucose units [1]. These enzymes are of great significance in present day biotechnology with applications ranging from food, fermentation, textile to paper industries [2]. Although amylases can be derived from several sources, including plants, animals and microorganisms, microbial enzymes generally meet industrial demands. Today a large number of microbial amylases are available commercially and they have almost completely replaced chemical hydrolysis of starch in starch processing industry [2].

The composition and concentration of fermentation media greatly affect the growth and production of extracellular amylase in bacteria [3, 4, 5] yeasts [6, 7] and *Aspergillus* sp. [8]. Shinke *et al.* [9] reported that medium composition affects amylase production as well as sporulation in *Bacillus cereus*. The nature and amount of nitrogen source in the culture medium also affect the growth of the bacterium and amylase production [3, 6]. Besides carbon and nitrogen, sodium and potassium salts [3, 9], metal ions [3] and detergents [7] are known to affect amylase production and the growth of the organism. The growth medium for amylase production should obviously be one that provides a good yield of extracellular amylase.

Many factors such as temperature, aeration and inoculum concentration affect the growth of microorganisms and enzyme production [10]. It is difficult to determine the key factors of the fermentation medium and to optimize the cultural conditions with traditional methods (single dimensional) since it is laborious, time-consuming and incapable of reaching the true optimal point due to the interactions between variables. Response Surface Methodology (RSM), a better experimental strategy for seeking optimal conditions for multi-variable system, have been successfully employed for optimizing the medium composition and operating conditions in many bioprocesses [11, 12]. The aim of this study was to determine the optimal cultural conditions employing RSM for improving α - amylase production by *Bacillus subtilis* HB04

MATERIALS AND METHODS

Microorganisms and media

The new strain *Bacillus subtilis* HB04 was isolated from the enteric gut of Honey bee and the culture was maintained at 4 ^{o}C and subcultured every two weeks. Starch broth medium containing (g/L): soluble starch, 20.0; yeast extract, 4.0; peptone, 10.0; $MgSO_4.7H_2O$, 0.5; NaCl, 0.5; $CaCl_2$, 0.2; was prepared for the production of amylase. The pH of the medium was adjusted to 7.0 with 1N NaOH or 1 N HCl and was autoclaved at 121°C for 15 min.

Cultural conditions

Five ml starch broth was inoculated with 1 ml of inoculum and was incubated at 30 °C for 18 h. This 5 ml of 18 h old cultures was then transferred into 95 ml of sterile starch broth medium and was incubated for 30°C for 24 h. After incubation the crude enzyme (α - amylase) was obtained by centrifugation of the culture broth at 10,000 x g for 10 min and this Cell Free Filtrate (CFF) was stored at -20 °C.

α - Amylase Enzyme assay

α - Amylase production was assayed in terms of amylase activity exhibited by the culture supernatant in the enzyme assay. The reaction mixture containing 0.1 ml of crude enzyme and 1.0 ml (1.0 %) solution of soluble starch in 50 mM Phosphate buffer (pH 7.5) was incubated at 50 ^{o}C for 5 minutes. The reaction was stopped by addition of 1.0 ml of 1 N NaOH. The

level of α amylase activity was determined by measuring the reducing sugar (glucose) released from soluble starch [13]. One unit of amylase activity was determined by measuring the amount of enzyme which liberates 1 µmol of reducing sugar as glucose per minute under the conditions of assay.

Experimental design

Screening of important nutrient components using Plackett – Burman design

This study was done by Plackett - Burman design for screening medium components with respect to their main effects and not their interaction effects [14] on enzyme production by *Bacillus subtilis* HB04. The medium components were screened for eleven variables at two levels, maximum (+) and minimum (-). According to the Plackett – Burman design, the number of positive signs (+) is equal to (N+1) / 2 and the number of negative signs (-) is equal to (N-1) / 2 in a row. A column should contain equal number of positive and negative signs. The first row contains (N+1) /2 positive signs and (N-1) / 2 negative signs and the choice of placing the signs is arbitrary. The next (N-1) rows are generated by shifting cyclically one place (N-1) times and the last row contains all negative signs. The experimental design and levels of each variable is shown in Table 1. The medium was formulated as per the design and the flask culture experiment for amylase was assayed as described earlier. Response was calculated at the rate of amylase production and expressed as U/mL. All experiments were performed in triplicate and the average of the rate of amylase production was considered as the response.

The effect of each variable was calculated using the following equation:

$$E = (\sum M_{+} - \sum M_{-}) / N$$

where E is the effect of tested variable, M_+ and M_- are responses (amylase activities) of trials at which the parameter was at its higher and lower levels respectively and N is the number of experiments carried out.

The standard error (SE) of the variables was the square root of variance and the significance level (p – value) of each variables calculated by using Student's t – test.

$$t = E_{xi} / SE$$

where E_{xi} is the effect of tested variable. The variables with higher confidence levels were considered to influence the response or output variable.

Optimizaion of concentrations of the selected medium components using RSM

RSM is an emprical statistical modeling technique employed for muliple regression anaysis using quanitative data obtained from factorial design to solve multivariable equations simultaneously [15]. The screened medium components affecting enzyme production were optimized using central composite design (CCD) [16, 17].

According to this design, the total number of treatment combinations is $2k + 2k + n0$ where 'k' is the number of independent variables and $n0$ the number of repetitions of the experiments at the center point. For statistical calculation, the variables X_i have been coded as x_i according to the following transformation:

$$x_i = X_i - X_0 / \delta X$$

Where x_i is dimensionless coded value of the variable X_i, X_0 the value of the X_i at the center point, and δX is the step change. A 2k-factorial design with eight axial points and six replicates at the center point with a total number of 20 experiments was employed for optimizing the medium components.

The behavior of the system was explained by the following quadratic equation:

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j$$

Where Y is the predicted response, β_0 the intercept term, β_i the linear effect, β_{ii} the squared effect, and β_{ij} is the interaction effect. The regression equation was optimized for maximum value to obtain the optimum conditions using Design Expert Version 7.1.5 (State Ease, Minneapolis, MN).

Validation of the experimental model

The statistical model was validated for α - amylase production under the conditions predicted by the model in flask conditions. Samples were withdrawn at the desired intervals and α amylase assay was determined as described above.

RESULTS AND DISCUSSION

Plackett – Burman design

The influence of eleven medium factors namely pH, temperature, agitation, inoculum concentration, incubation time, starch, peptone, KH_2PO_4, yeast extract, NaCl and $CaCl_2$ in the production of α - amylase was investigated in 12 runs using Plackett – Burman design. Table 1 represents the Plackett–Burman design for 11 selected variables and the corresponding response for α - amylase production in 12 runs. Variations ranging from 62.43 to 253.66 U / mL in the production of α - amylase in the 12 trials were observed by Plackett – Burman design.

α - amylase production by *Bacillus subtilis* HB04 were subjected to response surface methodology and found inoculum concentration, incubation time and yeast extract as positive factors. These findings support other investigations of the same enzyme belonging to other microbial species [18, 19]. They were identified as most significant variables in α - amylase production and selected for further optimization.

The inoculum level was also an important factor for the production of α - amylase. Various inoculum levels (1, 2, 3, 4 and 5%) were used to study their effect on amylase production. The higher enzyme production (288.06 U/mL) was obtained at 3% inoculum level. However Sen reported a 10% inoculum level for the production of enzyme by *Bacillus licheniformis* S40 [20]. With the increase in inoculum level, the production of enzyme declined due to exhaustion of nutrients in the fermentation mash.

Incubation time had a significant role in enzyme production. Extended period of incubation might lead to the decomposition of enzyme due to interaction with other components in the media [21]. In this study, maximum amylase production was observed at 33 h by *Bacillus subtilis* HB04.

Yeast extract is the key nutrient material which controls the biosynthesis of this enzyme. This fact has also been suggested previously during other enzyme production experiments on nitrogen repression effects [22, 23]. McTigue *et al.* [24] reported that the results of enzyme yield are probably due to the excessive amount of the yeast extract, which may inhibit the production of enzyme when concentration of yeast exceeds a critical value [25, 26]; this inhibition may also be caused by the simultaneous addition of two complex nitrogen sources [24].

Yeast extract of 3 g /L showed maximum amylase production by *Bacillus subtilis* HB04. On the basis of analyzing the results, it may be concluded that microorganisms require a low level of nitrogen in order to produce enzymes because nitrogen may be a limiting factor [26]. Enzyme production was found to be highest with addition of yeast extract in the basal medium while supplementation of inorganic nitrogen sources reduced the enzyme production [27].

Statistical analysis of the Plackett – Burman design demonstrates that the model F value of 0.72 is significant. The values of $p < 0.05$ indicate model terms are significant (Table 2).

Regression analysis was performed on the results and first order polynomial equation was derived representing α - amylase production as a function of the independent variables.

$$\text{Amylase} = +157.00 + 26.00D + 10.33E + 6.33\ J$$

The magnitude of the effects indicates the level of the significance of the variable on α - amylase production. Consequently, based on the results from this experiment, statistically significant variables *i.e.* inoculum concentration, incubation time and yeast extract with positive effects were further investigated with central composite design to find the optimal range of these variables.

Central composite design

This is a very useful tool to determine the optimal level of medium factors and their interaction. Based on Plackett - Burman design, inoculum concentration, incubation time and yeast extract were selected for further optimization using response surface methodology. To examine the combined effect of these factors on α - amylase production, a central composite design (CCD) was employed within a range of -2 to +2 in relation to production of α - amylase. The results obtained from central composite design are given in Table 3.

The results obtained from the central composite design were fitted to a second order polynomial equation to explain the dependence of α - amylase production on the medium components.

The analysis of variance of the quadratic regression model suggested that the model is very significant as was evident from the Fisher's F – test (Table 4).

The model's goodness of fit was checked by determination coefficient (R^2). In this case, the value of R^2 value (0.9611) closer to 1 denotes better correlation between the observed and predicted responses. The coefficient of variation (CV) indicates the degree of precision with which the experiments are comapared. The lower reliability of the experiment is usually indicated by high value of CV. In the present case a low CV (5.48) denotes that the experiments performed are highly reliable. The *p* values denotes the significance of the coefficients and also important in understanding the pattern of the mutual interactions between the variables.

The fitted response for the above regression model was plotted in Figure 1. 3D graphs were generated for the pair wise combination of three factors for α - amylase production. Graphs highlight the roles played by various factors and also to emphasize the roles played by the physical constraints.

Validation of the model

The maximum experimental response for α - amylase production was 288.06 U/mL whereas the predicted value was 283.23 U/mL indicating a strong agreement between them. The optimum values of the tested variables are inoculum concentration 3%, incubation time 33h and yeast extract 3 g/L. In an attempt to optimize industrial conditions for α - amylase production, scale – up study was carried out in a jar fermentor by using medium under optimum conditions. The maximum production of 292.34 U/mL α - amylase was achieved. The results are encouraging for optimization under pilot scale or industrial scale conditions.

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Table - 1
Plackett – Burman experimental design for evaluating factors influencing alpha amylase by *Bacillus subtilis* HB04

Run	A	B	C	D	E	F	G	H	J	K	L	α amylase U/ mL
1	6	20	200	5	96	5	3	2	4	0.1	0.2	231.76
2	4	20	200	1	96	20	3	6	4	0.5	0.05	62.43
3	6	40	0	5	96	20	3	2	2	0.5	0.05	165.93
4	6	40	200	1	6	5	6	2	4	0.5	0.05	129.32
5	6	40	0	1	6	20	3	6	4	0.1	0.2	54.99
6	4	40	200	5	6	5	3	6	2	0.5	0.2	163.47
7	4	40	200	1	96	20	6	2	2	0.1	0.2	215.58
8	4	40	0	5	96	5	6	6	4	0.1	0.05	253.66
9	4	20	0	1	6	5	3	2	2	0.1	0.05	248.11
10	6	20	0	1	96	5	6	6	2	0.5	0.2	78.16
11	6	20	200	5	6	20	6	6	2	0.1	0.05	235.26
12	4	20	0	5	6	20	6	2	4	0.5	0.2	251.67

A: pH B: Temperature (^{o}C)
C: Agitation (rpm) D:Inoculum concentration (%)
E: Incubation time (h) F: Starch(g/L)
G: Peptone (g/L) H: KH_2PO_4(g/L)
J: Yeast extract (g/L) K: NaCl (g/L) L: $CaCl_2$ (g/L)

Table – 2
Analysis of variance for α - Amylase production by *Bacillus subtilis* HB04

Source	Sum of square	DF	Mean square	F-Value	*p* - Value
Model	1260.603233	4	315.151	0.75245638	0.0067
D-Inoculum Conc.	882.882075	1	887.882	2.85500341	0.0098
E-Incubation time	44.352075	1	54.3521	0.11152465	0.0482
J-Yeast extract	21.147075	1	25.1471	0.05317497	0.0242
Residual	2783.819658	7	385.689		
Cor Total	4044.422892	11			

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Table – 3
Experimental plan for optimization of alpha amylase production using central composite design

Run	A: Ino. Conc (%)	B: Incu. time (h)	C: Yeast (g/L)	α - amylase (U/mL)	
				Experiment	Predicted
1	-1	-1	-1	76.93	43.947332
2	1	-1	-1	98.99	108.61863
3	-1	1	-1	160.13	151.75536
4	1	1	-1	201.13	216.42666
5	-1	-1	1	73.45	55.14373
6	1	-1	1	93.54	98.815031
7	-1	1	1	197.15	183.95176
8	1	1	1	198.04	227.62306
9	-1	0	0	0	41.165542
10	1	0	0	141.64	132.27045
11	0	-1	0	0	18.7332
12	0	1	0	214.94	217.70279
13	0	0	-1	211.84	218.30298
14	0	0	1	241.49	237.13301
15	0	0	0	278.15	283.23508
16	0	0	0	286.05	283.23508
17	0	0	0	288.06	283.23508
18	0	0	0	281.82	283.23508
19	0	0	0	276.81	283.23508
20	0	0	0	291.5	283.23508

$$Y = +283.24 + 27.09A + 59.15B + 5.60C + 0.000AB - 5.25AC + 5.25BC - 69.48A^2 - 58.34B^2 - 19.63C^2$$

where Y is the predicted response for α - amylase production, A, B and C are the coded values of inoculum concentration, incubation time and yeast extract respectively.

Table – 4
ANOVA for the experimental results of the central composite design (quadratic model)

Source	Sum of square	DF	Mean square	F – Value	*p* - Value
Model	167721.6852	9	18635.7	27.432675	< 0.0001
A-Inoculum conc.	10019.11162	1	10019.1	14.748596	0.0033
B-Incubation time	47788.02638	1	47788	70.346185	< 0.0001
C-Yeast extract	428.0034861	1	428.003	0.6300409	0.4458
AB	0	1	0	0	1.0000
AC	220.5	1	220.5	0.3245862	0.5814
BC	220.5	1	220.5	0.3245862	0.5814
A^2	69568.64039	1	69568.6	102.40826	< 0.0001
B^2	49053.58031	1	49053.6	72.209139	< 0.0001
C^2	5552.214015	1	5552.21	8.1731158	0.0170
Residual	6793.264823	10	679.326		
Lack of Fit	6617.93149	5	1323.59	37.744856	0.0006
Pure Error	175.3333333	5	35.0667		
Cor Total	174514.95	19			

CV – 5.48; R^2 – 0.9611

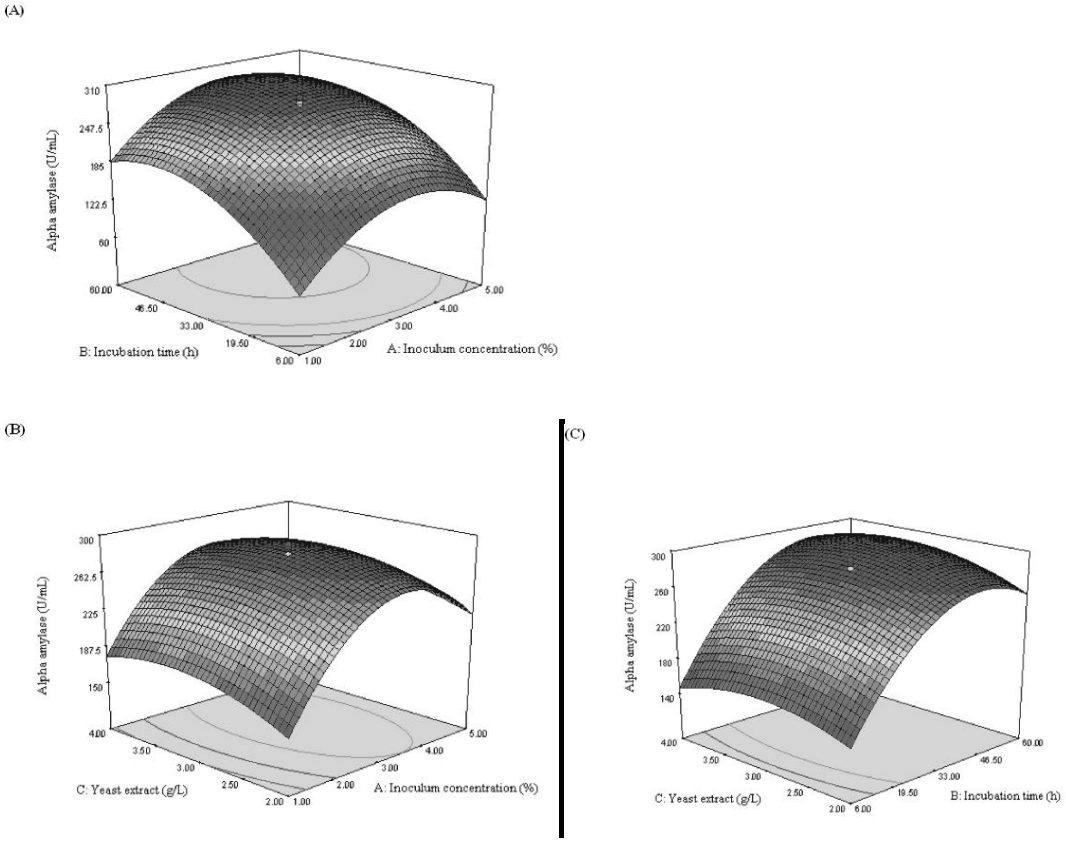

Figure – 1
Three dimensional response surface plot for the effect of (A) inoculum concentration, incubation time; (B) inoculum concentration, yeast extract; (C) incubation time, yeast extract

CONCLUSION

In the optimization of bioprocess variables for α - amylase production by *Bacillus subtilis* HB08, the combination of PBD with CCD is effective and reliable in selecting the statistically significant factors and finding the optimal concentration of those factors in fermentation medium. The work demonstrates the use of a central composite design in determining the optimum conditions leading to the maximum yield of enzyme production. This methodology could therefore be successfully employed to any process, where an analysis of the effects and interactions of many experimental factors are required. Thus, smaller and less time consuming experimental designs will generally suffice for the optimization of fermentation process.

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